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Water chlorination is a standard treatment for ensuring the safety of public drinking water. One drawback to this beneficial practice is the generation of drinking water disinfection by-products (DWDB), some of which have been implicated as causing adverse human health outcomes (Savitz et al. 1995; Waller et al. 1998; Weisel et al. 1996). In this article we report the results of 96 h developmental toxicity tests with embryos of the South African clawed frog Xenopus laevis used to evaluate four individual DWDB; bromodichloromethane (BDCM), sodium chlorate, chloroform, and dibromoacetic acid (DBAA). These chemicals were selected for testing based on their potential for human hard and as representatives of byproducts of different disinfection processes. Endpoints measured included embryo mortality (LC50), embryo malformation (EC50mal), embryo immobilization (EC50imm), no observed adverse effect level (NOAEL), and the minimum concentration to inhibit growth (MCIG).

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Developmental toxicity; FETAX; water disinfection by-products; frogs; Xenopus laevis; embryo malformations; embryo mortality; bromodichloromethane; sodium chlorate; chloroform; dibromoacetic acid

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Developmental Toxicity of Drinking Water Disinfection By-Products to Embryos of the African Clawed Frog (*Xenopus laevis*)

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Water chlorination is a standard treatment for ensuring the safety of public drinking water. One drawback to this beneficial practice is the generation of drinking water disinfection by-products (DWDB), some of which have been implicated as causing adverse human health outcomes (Savitz et al. 1995; Waller et al. 1998; Weisel et al. 1996). In this article we report the results of 96 h developmental toxicity tests with embryos of the South African clawed frog *Xenopus laevis* used to evaluate four individual DWDB: bromodichloromethane (BDCM), sodium chlorate, chloroform, and dibromoacetic acid (DBAA). These chemicals were selected for testing based on their potential for human harm and as representatives of byproducts of different disinfection processes. Endpoints measured included embryo mortality (LC_{50}), embryo malformation (EC_{50mal}), embryo immobilization (EC_{50imm}), no observed adverse effect level (NOAEL), and the minimum concentration to inhibit growth (MCIG).

MATERIALS AND METHODS

Test methods followed the Frog Embryo Teratogenicity Assay – *Xenopus* (FETAX) (Fort et al., 1993). Embryos were cultured in FETAX solution as described in the ASTM Standard Guide (ASTM, 1998). FETAX solution was prepared by adding 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4{\cdot}2H_2O$, and 75 mg $MgSO_4$ per liter of distilled water. The final pH of the solution was 7.6 to 7.9. Testing with BDCM and DBAA was conducted with oxygenated FETAX-AB used as the media since half of the test jars contained a metabolic activation system (MAS) component requiring considerable biological oxygen demand. The MAS results were inconsistent and are not reported here. South African clawed frog (*Xenopus laevis*) embryos for the FETAX assays were supplied from in-house cultures. Adult frogs were induced to mate by injecting 500 IU (males) and 750 IU (females) of human chorionic gonadotropin (hCG) (Sigma Chemical Co., St. Louis, MO) into the dorsal lymph sac of the frogs. Mating pairs were bred in the dark at 24 ± 2°C in FETAX solution. Amplexus typically occurred 4-6 h after injecting hCG; egg deposition occurred 9-12 h following hCG injection. Embryos were staged to obtain gastrulae between normal stage8 blastulae and normal stage 11 gastrulae. The embryos were de-

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jellied in 2% L-cysteine in FETAX solution pH adjusted to 8.1 rinsed three times with FETAX solution, resuspended in FETAX solution, staged, and then assigned randomly to test vessels. Bromodichloromethane (CAS # 75-27-4) and chloroform (CAS # 67-66-3) of 98+% purity were obtained from Aldrich Chemical Company, Milwaukee, WI. Stock solutions of BDCM were prepared daily in oxygenated FETAX-AB solution with target nominal concentrations of 25, 50, 75, 150, 250, 350, 450, and 550 mg/L for all three tests. Stock solutions of chloroform were prepared daily in FETAX solution. Target nominal concentrations were 25, 50, 75, 100, 125, 150, 175, 200, 250, and 300 mg/L for all three tests. Chloroform tests were conducted in 225 mL glass screw top jars sealed with Teflon-lined jar lids; BDCM in 115 mL jars. Samples were taken for chemical analysis from the stock solutions and each test concentration at the time of static renewal (T_0), and again 24 h later (T_{24}). Chloroform and BDCM samples were analyzed by headspace analysis using a Hewlett Packard 6890 gas chromatograph equipped with a flame ionization detector and interfaced to a Hewlett Packard model 7694 headspace sampler (Hewlett Packard, Avondale, PA). The instrumental method detection limit for chloroform and BDCM was 10 mg/L. The average percent recoveries for chloroform and BDCM were 94% and 97%, respectively. Mean measured values of chloroform averaged within 1% of nominal concentrations, while mean measured values of BDCM for each test averaged within 15% of nominal concentrations.

Sodium chlorate (CAS # 7775-09-9) was obtained from Aldrich Chemical Company, Milwaukee, WI. A stock solution was prepared in FETAX solution. Target nominal sodium chlorate concentrations for all tests ranged from 1000 to 8000 mg/L sodium chlorate with eight treatment levels in 1000 mg/L increments. Samples were collected at time of static renewal and analyzed using a Dionex 500 series Ion Chromatograph (IC) (Dionex, Sunnyvale, CA). The instrumental detection limit for sodium chlorate was 1 mg/L. The average percent recovery for sodium chlorate for all the assays was 101%. Mean measured sodium chlorate values averaged within 1% of nominal concentrations.

Dibromoacetic acid (DBAA) (CAS #631-64-1) was obtained from Aldrich Chemical Company, Milwaukee, WI. A stock solution was prepared in FETAX and pH neutralized. All test concentrations were prepared fresh daily by diluting the stock with FETAX-AB. Using a 0.5 dilution factor, the ten nominal DBAA concentrations ranged from 25 to 12,800 mg/L for all three tests. Both DBAA and sodium chlorate were tested in 60mm glass Petri dishes. Samples were collected in glass scintillation vials from the stock and each test concentration at time of static renewal. Samples were analyzed using a Hewlett Packard 1050 series Liquid Chromatograph (Hewlard Packard, Avondale, PA). The instrumental detection limit for DBAA was 4 mg/L. The average percent recovery for DBAA for all the assays was 102%. Mean measured DBAA values averaged within 2% of nominal concentrations.

The ASTM "Standard Guide for Conducting the Frog Embryo Teratogenesis

Assay – *Xenopus* (FETAX)" (ASTM, 1998) and the "Atlas of Abnormalities" (Bantle et al. 1998) were used to ensure reproducibility among testing procedures. Research was conducted in compliance with the Animal Welfare Act and in accordance with other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals,* National Academy Press, Washington, DC, 1996, for facilities that are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International.

Two replicates of 25 embryos per replicate were used for each of the chloroform, DBAA, and sodium chlorate test concentrations. Because the BDCM test was performed in smaller jars, the numbers of embryos were reduced to 15 per replicate, while the control treatments remained at 25 embryos for each of the four replicates. Triplicate 96 h tests were run on all four compounds. The tests were conducted at 24 ± 2°C in the dark in a constant temperature environment incubator. Test solutions were replaced every 24 ± 2h with freshly made solutions. Mortality, judged by the absence of heartbeat, was noted daily, and all dead embryos were removed during daily test solution renewals. LC_{50} values were calculated using probit analysis based on the number of surviving embryos at 96 h. Malformations of surviving embryos were observed with a dissecting microscope and recorded at the conclusion of the 96 h test. EC_{50mal} values were calculated based on the percent of surviving embryos with malformations at 96 h. Embryo immobilization was noted during chloroform testing only and the percent of immobilized embryos was recorded and reported as EC_{50imm}. The frog embryos were euthanized with MS-222 and preserved in 3% (w/v) formalin. Lengths were measured electronically on formalin-fixed embryos using digitized computer software (Bioquant, R and M Biometrics, Inc., Nashville, TN). No observed adverse effect levels (NOAEL) and minimum concentration to inhibit growth (MCIG) were calculated from embryo length measurements. Embryo length measurements were analyzed using normal regression and analysis of variance. Kolmogorov-Smirnov tests were used to check the assumption of normality. All computations are made using S-Plus statistical software. NOAEL and MCIG were calculated from embryo length measurements through a series of two-sample T-tests adjusted for multiple comparisons.

RESULTS AND DISCUSSION

At BDCM exposures ≥250 mg/L, all embryos were severely malformed (Table 1). Malformations that occurred with the highest frequency include notochord maldevelopment, craniofacial defects, and cardiac edema. The EC_{50} for BDCM malformations (62-72 mg/L) was at a much lower concentration than the LC_{50} (396-440 mg/L) (Table 2). Sodium chlorate was much less toxic than BDCM, and there was less separation between the LC_{50} and EC_{50}. Embryo malformations at sodium chlorate concentrations ≤ 5000 mg/L were within the background rate of ≤10%. In contrast to BDCM, the effects of sodium chlorate appeared to be directed almost exclusively toward gut malformation. Gut malformations of 35%

Table 1. Developmental toxicity in FETAX: Summary of embryo malformations.

Malformation	% Embryos Affected[a]											
	BDCM (mg/L)				Sodium Chlorate (mg/L)			Chloroform (mg/L)				
	50	75	150	≥250	5000	6000	≥7000	100	125	150	175	≥200
Gut	0	0	0	-[b]	35	96	-[b]	0	0	0	0	-[b]
Cardiac	0	0	23	-[b]	0	0	-[b]	19	25	26	16	-[b]
Craniofacial	0	0	41	-[b]	0	0	-[b]	16	24	26	16	-[b]
Notochord	11	41	-[b]	-[b]	0	0	-[b]	18	18	12	-[b]	-[b]
Severe	0	0	58	100	0	0	100	18	0	74	84	>97

[a] Embryos in the controls and concentrations not shown were below the background malformation level of 10% and had no severe malformations.
[b] These malformations may be present but fall into the "severe" classification when organ system is no longer recognizable.

Table 2. Developmental toxicity in FETAX: Summary of statistical analyses.

96 h Endpoint	BDCM[a]	Sodium Chlorate[a]	Chloroform[a]
LC_{50} (mg/L)	424 (435, 440, 396)	5778 (6475, 5635, 5225)	-[b]
EC_{50imm} (mg/L)	-	-	111 (69, 132, 133)
EC_{50mal} (mg/L)	64 (62, 72, 58)	4865 (5064, 5362, 4170)	110 (79, 120, 131)
NOAEL (mg/L)	-[c](<15, 21, 21)	-[c] (2883, 2960, 1043)	-[c] (21, 50, <25)
MCIG (mg/L)	-[c] (15, 21, 45)	-[c] (3897, 3966, 1996)	-[c] (46, 76, 25)

Abbreviations: LC_{50} = 50% mortality (probit analysis); EC_{50imm} = 50% immobilization (probit analysis); EC_{50mal} = 50% malformation (probit analysis); NOAEL = no observed adverse effect level (length); MCIG = minimum concentration to inhibit growth (length)
[a] Mean of 3 individual test in ().
[b] Mortality was nonlinear so an EC_{50} for immobilization was calculated instead.
[c] Could not be pooled due to inequality of the slopes and intercepts (linear regression model)

and 96% prevalence occurred at 5000 and 6000 mg/L sodium chlorate, respectively. No other malformations occurred above background rates in the 5000 to 6000 mg/L concentration range. All surviving embryos at 7000 and 8000 mg/L sodium chlorate had the severe malformations.

Chloroform toxicity levels were comparable to BDCM, but $LC_{50}S$ could not be calculated for chloroform because the mortality rate was nonlinear, while immobilization (EC_{50imm}) increased with increasing chloroform concentrations (Table 2). Narcosis and respiratory depression leading to immobilization are known consequences of chloroform intoxication (WHO, 2004). Since immobilization preceded the spike in mortality, it is possible that immobilized embryos take up less chloroform, resulting in delayed mortality at higher concentrations. Chloroform induced characteristic malformations of cardiac edema, notochord maldevelopment, and craniofacial defects. DBAA did not induce malformations until concentrations approached the 96-h LC_{50}. At these levels, DBAA induced malformation of the gut. Incidence of malformations in DBAA-treated embryos was $\leq$15% for all treatments, except for a 64% prevalence of malformations in one of the replicate tests at the highest test concentration. Similarly, growth was inhibited only at high levels of exposure. Due to the relative non-toxic nature of DBAA in these experiments, the data are not presented here, but the test concentrations ranged from 25 to 12,800 mg/L.

The life-long exposure of most individuals in the developed world to disinfected drinking water has raised concerns about effects of chronic exposure to these chemicals. Although some epidemiology studies have implicated DWDB in adverse health outcomes, the literature to date has been conflicting in demonstrating a consistent pattern of association of exposure of pregnant women to trihalomethanes in drinking water with resultant spontaneous abortions, stillbirths, or congenital anomalies. In a three year long epidemiological study, human consumption of $\geq$ 5 glasses per day of cold tap water containing $\geq$ 75 ug/L trihalomethanes was associated with an increased incidence of spontaneous abortions (Waller et al. 1998). Of the four trihalomethanes studied, only BDCM human consumption $\geq$ 18 μg/L was suggested to be individually correlated with spontaneous abortions. Pregnant women in Nova Scotia exposed to 100 μg/L or more of trihalomethanes in the drinking water had an increased risk of stillbirths, but with little or no evidence of increased risk of congenital anomalies, with the possible exception of chromosomal abnormalities (Dodds et al, 1999). Epidemiological studies done in California found no clear pattern of association between exposure of pregnant women to trihalomethanes in municipal water supplies and the occurrence of congenital malformations (Shaw et al. 2003). The levels of trihalomethanes reported in these human studies were in the concentration range of 100 μg/L, whereas the levels used in the FETAX study reported here were at levels of 50 mg/L and greater.

Exposure to either of the trihalomethanes tested in this study resulted in adverse developmental effects. Both chloroform and BDCM appeared to inhibit growth at

low concentrations and caused developmental defects in the notochord and face as well as cardiac edema. The developmental effects of sodium chlorate were restricted to retardation of gut coiling at very high concentrations and growth inhibition at slightly lower concentrations. DBAA did not produce morphological defects or growth inhibition except at concentrations approaching lethal exposure. Observed adverse effects on frog embryos of these four chemicals occurred at concentrations many orders of magnitude above levels of chloroform, BDCM, DBAA, and sodium chlorate commonly found in drinking water [22.4, 6.7, <0.25, and 230 ug/L, respectively] (Borum et al. 1998).

Based on 96-h LC_{50}S for the Japanese medaka fish, Toussaint et al. (2001) found the relative order of acute toxicity to be BDCM>chloroform>DBAA>sodium chlorate. Our frog embryo lethality data followed a similar pattern, except that sodium chlorate was more acutely toxic than DBAA. Studies in mammals also suggest that BDCM is more biologically active than chloroform. Our results are in agreement with these general findings in that BDCM was more developmentally toxic to frog embryos than chloroform.

Despite different routes of exposure between frog embryos and rodents, some comparisons may be made if the characteristic malformations elicited are viewed generically. Neither sodium chlorate nor DBAA were found to be teratogenic in rodents. (IARC 2000; Christian et al. 2002) Our frog embryo results show that malformations do not occur until sodium chlorate or DBAA concentrations approach lethal levels. Skeletal abnormalities seen in rats exposed to BDCM (Christian et al. 2000) may compare with notochord maldevelopment seen in our *Xenopus* embryos. Chloroform results in rodents are species dependent, with mice pups developing cleft palates and rat fetuses having reduced weight gain (Ruddick et al. 1983). While we did not weigh our frog embryos, differential growth in replicate chloroform tests made data interpretation difficult, and facial abnormalities were typical of chloroform exposure.

Although adverse effects of the DWDB tested in this study occurred at much higher concentrations than those reported in drinking waters, our test results are consistent with other studies that rank the relative developmental toxicity of the trihalomethanes BDCM and chloroform as greater than either sodium chlorate or DBAA.

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